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Melanoma Antigen-Encoding Gene-1 Expression in Invasive Gastric Carcinoma: Correlation With Stage of Disease

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Background: A human melanoma antigen-encoding gene-1, MAGE-1 gene, may be linked to the neoplastic transformation. In the present study, we extended this association with human gastric carcinomas. Specifically, we focused on the relationship between MAGE-1 gene expression and the histologic stage of gastric carcinoma.

Methods: We used a reverse transcription-polymerase chain reaction assay (RT-PCR) to analyze the expression of the MAGE-1 gene in 38 endoscopic biopsy specimens from gastric carcinomas. We also studied the relationship between the expression of MAGE-1 gene and the genetic expression of several tumor invasion-related factors, including 72 kD type IV collagenase (MMP2), urokinase-type plasminogen activator (uPA), platelet-derived growth factor A (PDGF-A), and vascular endothelial growth factor (VEGF).

Results: Eleven of the 38 tumor samples (28.9%) expressed the MAGE-1 gene. MAGE-1 gene expression was present only in two of the 38 adjacent nontumor samples (5.3%). MAGE-1 gene expression in the 38 tumor samples was significantly correlated with the histological stage of disease ($P = 0.0008$), especially with the depth of histologically confirmed tumor invasion (t1 vs. t2 or greater, $P = 0.00048$). The expression of MAGE-1 gene correlated with the expression of MMP2 ($P = 0.0064$), uPA ($P = 0.0390$), and PDGF-A ($P = 0.00018$).

Conclusions: These data suggest that the MAGE-1 gene may be activated in gastric carcinomas during periods of their development or invasion. In addition, a relationship between MAGE-1 gene expression and expression of invasion-related factors has been demonstrated.

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KEY WORDS: RT-PCR; mRNA expression; tumor invasion

INTRODUCTION

Van der Bruggen et al. [1] have identified the human melanoma antigen-encoding gene-1, MAGE-1, which directs the expression of an antigen recognized in melanoma by autologous cytolytic T cells. Through cross-hybridization with MAGE-1, it has been demonstrated that the MAGE gene family consists of at least 12 closely related genes that are located on the long arm of chromosome X [2]. The MAGE gene, especially MAGE-1, is expressed at high levels in a number of tumors [3–5]. However, expression was absent in a large number of healthy tissues, with the exception of the testis and placenta [2,6]. These findings indicated that the MAGE-1

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TABLE I. Conclusive Stage Grouping of 38 Primary Gastric Carcinomas According to the General Rules for the Gastric Cancer Study of the Japanese Research Society for Gastric Cancer*

	n0	n1	n2	n3	P0H1 n2>	
t1 (m, sm)	Ia	Ib	II	IIIa		
t2 (mp, ss)	Ib	II	IIIa	IIIb	IVa	IVb
t3 (se)	II	IIIa	IIIb	IVa		
t4 (si)	IIIa	IIIb	IVa			
P1H0 t3>	IVa			2 or more of t4 (one organ), n3, P1, and H1 t4 (several organs), n4, P2,3, H2,3, M1		

*Abbreviations: t1: tumor invasion of mucosa (m), submucosa (sm), t2: tumor invasion of muscularis propria (mp) or subserosa (ss), t3: tumor invasion of serosa without invasion of adjacent structures (se), t4: tumor invasion of adjacent structures (si), M1: distant metastasis, P1: peritoneal dissemination, H1: hepatic metastasis.

gene may be linked to a transformation event and that MAGE-1 gene expression may be tumor-specific.

Recently, it was reported that MAGE-1 mRNA was expressed in two of seven Epstein-Barr virus-transformed B-cell lines and two of five breast cell lines, including a cell line established from normal breast epithelium [7]. Becker et al. [8] analyzed MAGE-1 mRNA expression in skin during wound healing. They demonstrated that the MAGE-1 gene was expressed in tumor-free human skin during the early stages of wound healing and suggested that the MAGE-1 gene was a normal gene that might be involved in the control of migration, proliferation, and differentiation during tissue repair. The process of wound healing is under the control of several growth factors. The growth factors involved in wound healing are also present in many human tumors [9,10]. These data suggested that the expression of MAGE-1 gene is not necessarily linked to transformation events and that a more extensive study of normal and tumor tissues is required for establishing the tumor-specific nature of MAGE-1 expression.

We collected relatively large numbers of biopsy specimens of gastric carcinoma in various histologic stages. In the present study, we demonstrated that the frequency of MAGE-1 gene expression in gastric carcinoma tissues increased as the histological stage advanced. The MAGE-1 gene may be activated in cases of gastric carcinoma during their differentiation, development, and especially invasion.

MATERIALS AND METHODS

Biopsy Samples

Tumor biopsy specimens were obtained during preoperative endoscopy of 38 patients with gastric carcinoma. All 38 primary gastric carcinoma surgical resection specimens were histologically classified using the General Rules for Gastric Cancer Study of the Japanese Research Society for Gastric Cancer. According to this classification, t1, t2, t3, and t4 correspond to tumor invasion of the mucosa or submucosa, muscularis propria or subserosa, the serosa without invasion of adjacent structures, and adjacent structures, respectively. N0, n1, n2, n3, and n4 signify the absence of nodal metastasis and the presence of group 1, group 2, group 3, and group 4 regional lymph node metastasis, respectively. M0 and M1 designate the absence and presence of distant metastasis, respectively. P0 and P1 indicate the absence and presence of peritoneal dissemination, respectively. H0 and H1 reflect the absence and presence of hepatic metastasis, respectively. Conclusive stage grouping is summarized in Table I. Histologic staging demonstrated 24 early cases (t1) and 14 advanced cases (t2 or greater).

Adjacent nontumor gastric tissue specimens were also collected. Tumor and nontumor specimens were collected simultaneously from each patient. Each specimen was divided into two blocks. One was minced in RNA lysis buffer on ice for extraction and subsequent analysis by reverse transcriptase-polymerase chain reaction (RT-PCR), whereas the other block was fixed in 10% formalin for histopathological examination.

Reverse Transcriptase-polymerase Chain Reaction (RT-PCR) and Gel Electrophoresis

Total RNA of each biopsy specimen was isolated by single step, guanidium thiocyanate-phenol- chloroform extraction [11]. Biopsy specimens maintained on ice were minced and homogenized manually in the presence of lysis buffer. RNA fractions were suspended in diethyl pyrocarbonate-treated water and quantitated by absorbance at 260 nm. RT-PCR was carried out according to the Perkin-Elmer/ Cetus protocol for reverse transcription (RT) of RNA and amplification of cDNA. Each RT reaction was carried out with 0.5 μg of RNA per sample. cDNA amplification of MAGE-1 was performed for 35

cycles (1 min at 94°C for denaturation and 3 min at 72°C for annealing and extension) [3]. cDNA amplification for vascular endothelial growth factor (VEGF) was performed according to the following parameters: 92°C for denaturation, 52°C for annealing, and 72°C for extension. Each step was 1 min in duration, and 10 cycles were performed. This amplification was followed by 25 cycles with an annealing temperature of 55°C. cDNA amplification for 72 kD type IV collagenase (MMP2), urokinase-type plasminogen activator (uPA), platelet-derived growth factor-A (PDGF-A), and β-actin were performed for 35 cycles with annealing temperatures of 58°C, 58°C, 60°C, and 58°C, respectively. Aliquots of PCR products (7.5 μl) were separated and visualized with ethidium bromide after electrophoresis in a 1.5% agarose gel in Tris acetate EDTA buffer at 100 V for 20 min. To confirm that only mRNA, but not contaminating DNA, was amplified in this assay, PCR was performed without RT for amplification of contaminating DNA. A specific band that indicates the amplification of contaminating DNA was not detected in any of the mRNA samples.

PCR Products Verification by Southern Blot

To verify that PCR amplification was specific for MAGE-1 mRNA, PCR products were transferred to nylon membranes and probed with a radiolabeled oligonucleotide complementary to sequences within the region flanked by a pair of the MAGE-1 primer. Blots were hybridized at 50°C with γ-^{32}P (γ-^{32}P-ATP; 7,000 Ci/mM; ICN Pharmaceuticals, Costa Mesa, CA) labeled on 5′ end by T4 polynucleotide-kinase (Boehringer Mannheim Biochemicals, Mannheim, Germany) for 18 h. Membranes were then washed for 10 min with 2 × SSC and 0.1% SDS, followed by 0.2 × SSC and 0.1% SDS at ambient temperature, and subjected to autoradiography.

Oligonucleotide Primers

PCR primers for MAGE-1, MMP2, uPA, PDGF-A, VEGF, and β-actin were designed to flank cDNA sequences that cross an intron-exon boundary in genomic DNA. Primer sequences were as follows (base pair fragment size predicted for each primer pair is noted):

MAGE-1 [3]: sense 5′-CGGCCGAAGGAACCTGACCC-3′, anti-sense 5′-GCTGGAACCCTCACTGGGTTGCC-3′ (421) MMP2 [12]:5′-AAGAGTGCATGGTGCATGAC, 3′-GCTGGTGCAGCTCTCATATT (377); uPA [12]:5′-AAGAGTGCATGGTGCATGAC, 3′-CTTGCGTGTTGGAGTTAAGC (317); VEGF [13]: 5′-ATGAACTTTCTGCTGTCTTGG, 3′-TCACCGCCTCGGCTTGTCACA (576 and 444); PDGF-A [14]: 5′-CCCCTGCCCATTCGGAGGAAGAGA, 3′-TTGGCCACCTTGACGCTGCGGTG (228)

The primers for VEGF were able to detect two of four different molecular species produced by alternative splicing of mRNA-VEGF165 and VEGF121 [15,16].

Statistics

Fisher's exact probability test was used for statistical analyses of total RNA contents between tumor and nontumor samples, total RNA contents between early and advanced stages of disease, total RNA content of between MAGE-1 gene expressing and nonexpressing specimens, MAGE-1 gene expression between tumor and nontumor specimens, MAGE-1 gene expression between t1 and t2–t4 specimens, and differential mRNA expression of invasion-related factors between MAGE-1 gene expressing and nonexpressing samples. Mann-Whitney's U-test was used for correlation analyses between MAGE-1 gene expression and histologic stage and between MAGE-1 gene expression and depth of tumor invasion.

RESULTS

In our study, total RNA content recovered from tumor and nontumor samples amounted to 6.87 ± 7.68 and 7.44 ± 7.50 μg/tissue specimen, respectively ($P = 0.722$). RNA recovered from early-stage cases and advanced-stage cases were 7.02 ± 6.69 and 6.69 ± 7.68 μg/tissue specimen, respectively ($P = 0.699$). Total RNA recovered from MAGE-1 gene expression positive cases and negative cases were 6.30 ± 8.94 and 7.29 ± 6.03 μg/tissue specimen, respectively ($P = 0.929$). β-actin gene was successfully amplified in every sample (data not shown).

MAGE-1 gene expression in tumor biopsy specimens was examined and compared with that from nontumor biopsy specimens. To verify the PCR products, those were transferred to nylon membrane, hybridized with a ^{32}P-labeled oligonucleotide primer complementary to a sequence internal to the PCR amplification primer, and scanned according to β emmision. Figure 1 shows representative data from six patients. The MAGE-1 gene was expressed in 11 cases (28.9%) from tumor specimens and in two cases (5.3%) from adjacent nontumor specimens ($P = 0.0063$) (Table II). Both of the two adjacent nontumor specimens were derived from patients with advanced carcinoma in whom the MAGE-1 gene was expressed in the tumor specimens. MAGE-1 gene expression was examined in various histological stages of disease. MAGE-1 gene was expressed in two of 24 patients with stage I disease, two of two patients with stage II disease, four of seven patients with stage III gastric carcinoma, and three of five patients with stage IV gastric carcinoma ($P = 0.0008$) (Table III). Since the data indicated that MAGE-1 gene expression may correlate with the stage of disease, the relationship between MAGE-1 gene expression and the depth of tumor invasion was examined. MAGE-1 gene was expressed in two

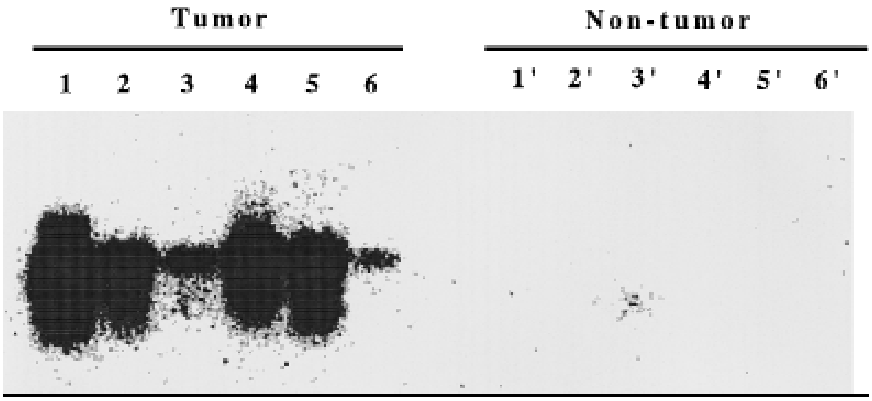

Fig. 1. Transcription of MAGE-1 gene in biopsy specimens from 6 patients with gastric carcinoma. RNA was extracted from endoscopic biopsy tumor specimens (lanes 1–6) and from simultaneously sampled nontumor tissues (lanes 1′–6′), reverse transcribed and assayed after 35 cycles polymerase chain reaction (PCR) for transcription of MAGE-1. PCR products were run in the agarose gel and transferred to nylon membrane, then hybridized with the internal antisence probes labeled ^{32}p-dCTP.

TABLE II. Gastric Carcinoma: Expression of MAGE-1 Gene by Tumor and Nontumor Specimens*

	Tumor specimens	Nontumor specimens
MAGE-1 (+)	11[a]	2
MAGE-1 (−)	27	36
% positive	28.9%	5.3%

*RNA was prepared from tumor or adjacent nontumor specimens as described in Materials and Methods and used for RT-PCR analysis. Positive specimens gave a consistent 421-base pair band for MAGE-1 in repeated experiments, whereas negative specimens did not.
[a]$P = 0.0063$ by Fisher's exact probability test.

TABLE III. Gastric Carcinoma: Expression of MAGE-1 Gene by Tumor Specimens in Different Stages of Disease*

	Stage[a]			
	I	II	III	IV
MAGE-1 (+)	2	2	4	3
MAGE-1 (−)	22	0	3	2
% positive	24%	100%	57.1%	60.0%

*All 38 primary gastric carcinoma surgical resection specimens were histologically classified using the General Rules for Gastric Cancer Study of the Japanese Research Society for Gastric Cancer (see Table I).
[a]$P = 0.0008$ by Mann-Whitney's U-test.

TABLE IV. Gastric Carcinoma: Expression of the MAGE-1 Gene in Different Depth of Tumor Invasion*

	Depth of tumor invasion[a]			
	t1	t2	t3	t4
MAGE-1 (+)	2 (0)	3 (0)	5 (2)	1 (0)
MAGE-1 (−)	22 (24)	1 (4)	3 (6)	1 (2)

*Tumor and adjacent nontumor specimens were collected simultaneously from each patient. t1, t2, t3, and t4 correspond to tumor invasin of the mucosa or submucosa, muscularis propria or subserosa, the serosa without invasion of adjacent structures, and adjacent structures, respectively.
[a]$P = 0.0006$ by Mann-Whitney's U-test.
( ) = nontumor specimens.

of 24 t1 specimens, three of four t2 specimens, five of eight t3 specimens, and one of two t4 specimens (Table IV). The proportion of MAGE-1 positive samples correlated with the depth of invasion(t) ($P = 0.0006$). When the expression was compared between t1 cases and t2–t4 cases, the difference was statistically highly significant ($P = 0.00048$).

Correlation analyses between MAGE-1 gene expression and several factors, including lymph node metastasis (n), tumor size, and histologic type, were performed. A relatively significant correlation between MAGE-1 gene expression and the level of n was found (n0 vs n1 or greater; $P = 0.011$) (Table V). The relationship between MAGE-1 gene expression and tumor size (< 20 mm vs > 20 mm) also was analyzed (Table VI). No significant correlation was found ($P = 0.087$). No significant correlation was found between MAGE-1 gene expression and histologic types (well-differentiated vs. poorly differentiated; $P = 0.159$) (Table VI). In this study, well-differentiated types consisted of well-differentiated and papillary adenocarcinomas. Poorly differentiated types included moderately differentiated and poorly differentiated adenocarcinomas and signet ring cell carcinomas.

It has been demonstrated that several invasion-related factors, such as protease and PDGF, play important roles in tumor invasion [10,17–19]. We analyzed the correlation between MAGE-1 gene expression and expression of invasion-related factors, including MMP2, uPA, VEGF, and PDGF-A. MAGE-1 gene expression showed a significant correlation with gene expressions of MMP2 ($P = 0.0064$), uPA ($P = 0.0390$), and PDGF-A ($P = 0.00018$) (Table VII). However, no significant correlation was found between MAGE-1 gene expression and other factors-related gene expression, including cyclin

TABLE V. Expression of MAGE-1 Gene by Tumor Specimens in Absence and Presence of Lymphnode Metastasis in Gastric Carcinoma*

	Lymph node metastasis[a]	
	n0[b]	n1<[c]
MAGE-1 (+)	4	7
MAGE-1 (−)	22	5
% positive	15.4%	58.3%

*n0 and n1< signify the absence and presence of nodal metastasis, respectively.
[a]$P = 0.011$ by Fisher's exact probability test.
[b]n0: absence of nodal metastasis.
[c]n1<: presence of nodal metastasis.

TABLE VI. Gastric Carcinoma: Expression of MAGE-1 Gene by Tumor Specimens in Different Tumor Size and Different Histologic Types*

	Size (mm)[a]		Histological type[b]	
	20>	20<	well diff.	poorly diff.
MAGE-1 (+)	2	9	1	9
MAGE-1 (−)	13	14	9	18
% positive	13.3%	39.1%	10.0%	33.3%

*Tumor specimens were divided into two groups by tumor size (20 mm> and 20 mm<) or histological types (well differentiated and poorly differentiated type). Well-differentated types consisted of well-differentiated and papillary adenocarcinomas, and poorly differentiated types consisted of moderately and poorly differentiated adenocarcinomas and signet ring cell carcinomas. One mucinous adenocarcinoma sample was excluded from this study.
[a]$P = 0.087$ by Fisher's exact probability test.
[b]$P = 0.159$ by Fisher's exact probability test.

D1 ($P = 0.7290$), cyclin E ($P = 0.7930$), TGF-β ($P = 0.3800$), and interleukin-10 ($P = 0.2520$).

DISCUSSION

We analyzed MAGE-1 mRNA expression in gastric carcinoma biopsy specimens. Our data indicated that MAGE-1 gene was expressed by a relatively large proportion of gastric carcinoma tissues (Table II and Fig. 1). In the present study, we focused on the relation between the expression of the MAGE-1 gene and the histologic stage of gastric carcinoma. The frequency of MAGE-1 gene expression increased as the histologic stage advanced (Table III). To our knowledge, this is the first study reporting MAGE-1 expression by biopsy specimens collected from relatively large numbers of gastric carcinoma tissue. The biopsy specimens had several characteristics: (1) samples were small in volume (2 mm in diameter) and all cases underwent endoscopic examination to confirm the malignant nature of the tumor prior to surgical resection; as a result, a bias favoring advanced cases in sample collection could be avoided, (2) the histological depth of specimens collected were uniform, and (3) most of the cases were not undergoing specific therapy for gastric carcinoma.

The MAGE gene family is composed of at least 12 closely related genes, the sequences of which show 64–85% homology with MAGE-1 [2]. It is difficult to distinguish MAGE-1 from other genes in the MAGE family because of this high degree of homology. In the present study, we specifically measured MAGE-1 gene expression by reverse transcription and polymerase chain reaction(RT-PCR) using oligonucleotide primers corresponding to unique MAGE-1 sequences [2,3]. The high frequency of expression of MAGE-1 is found in various histologic tumor types [1,3–5]. Since MAGE-1 gene expression is limited to the testes and placenta in normal tissues, it has been proposed that MAGE-1 gene may be linked to a neoplastic transformation event [2,6].

Our data also indicated a relatively large proportion of MAGE-1 gene expression (11/38, 28.9%) in gastric carcinoma samples and a low incidence (2/38, 5.3%) in nontumor samples (Table II). These data strongly suggest a tumor-specific role for MAGE-1 gene expression. It is notable that MAGE-1 gene expression was found in 2 of the 38 adjacent nontumor gastric samples. Both MAGE-1 mRNA-positive nontumor specimens were derived from patients with advanced cancer (t3) in whom MAGE-1 gene was also expressed in the tumor specimens (Table IV). Although histologic examination revealed that the two specimens did not contain visible carcinoma cells, the possibility that a small number of carcinoma cells, which could not be detected microscopically, may be present in the nontumor samples still exists. If this is the case, then the examination of MAGE-1 mRNA expression may be a highly sensitive tool for tumor cell detection. However, the MAGE-1 gene may be expressed not only by transformed cells, but also by normal epithelial cells under limited conditions in the stomach. The proportion of MAGE-1 gene expression in tumor samples was positively correlated with the histologic stage of disease, especially the depth of tumor invasion (Table IV). We speculate that the MAGE-1 gene is silent in normal gastric mucosa and activated during neoplastic transformation of epithelial cells during the process of invasion. MAGE-1 appears to be a stage-related gene. In contrast, Weber et al. [20] have reported that the MAGE-1 gene was expressed by melanoma tissue during all stages of disease. However, there is no data concerning MAGE-1 expression during various stages of disease in tumors of epithelial origin.

We cannot explain why the MAGE-1 gene is activated in gastric carcinoma cells during invasion. Recently, Becker et al. [8] have demonstrated that the MAGE-1 gene was expressed in normal skin during wound healing. Wound healing involves many different cell types in the process of migration, proliferation, differentiation, and protease-induced degradation of the extracellular

TABLE VII. Gastric Carcinoma: Relationship Between MAGE-1 Gene Expression and Expressions of Invasion-related Factors*

	Invasion-related factors							
	uPA		MMP2		VEGF		PDGF-A	
	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)
MAGE-1 (+)	10	1	11	0	4	7	9	2
MAGE-1 (−)	15	12	15	12	8	19	4	23
% positive	40.0%	7.7%	42.3%	0.0%	33.3%	26.9%	69.2%	8.0%
Significance[a]	$P = 0.039$		$P = 0.0064$		$P = 0.483$		$P = 0.00018$	

*Correlation analyses between MAGE-1 expression and expressions of several invasion-related factors, including 72 kD type IV collagenase (MMP2), urokinase-type plasminogen activator(uPA), platelet-derived growth factor-A(PDGF-A), and vascular endothelial growth factor (VEGF), were performed.
[a]Fisher's exact probability test.

matrix or angiogenesis [21]. Thus, Becker et al. [8] have suggested that the MAGE-1 gene is a normal gene and that the MAGE-1 gene is involved in cellular functions that are activated during inflammation, tissue repair, or angiogenesis. Several potent growth factors, such as platelet-derived growth factors (PDGF) [22] and epidermal growth factor (EGF) [23], are present during the process of wound healing. The biologic characteristics of tumor invasion are very similar to those of tissue injury and wound healing. The growth factors involved in wound healing are also present in many human tumors, especially during the advanced stages of disease. It is possible that MAGE-1 gene expression may be related to the expression of those proteins. We measured the expression of mRNAs of several tumor invasion-related factors, including MMP2, uPA, VEGF, and PDGF, in gastric carcinoma specimens. The expression of MAGE-1 gene showed a significant correlation with the mRNA expression of MMP2, uPA, and PDGF-A (Table VII). This result suggests an association between MAGE-1 gene expression and gene expressions of those cytokines. However, we have no data indicating that MAGE-1 gene expression is directly or indirectly influenced by these factors.

Although the function of MAGE-1 protein is not known, a computer search of Protein Sequence Database revealed a moderate homology between the MAGE protein and the mouse protein ''necdin'' [2]. Since there is considerable conservation of hydrophilic and hydrophobic regions between MAGE-1 and necdin, it has been suggested that the proteins produced by these genes may exert very similar functions [2]. Necdin is a protein expressed in neurally differentiated embryonal carcinoma cells, in the developing mouse brain during the early stages of neuronal generation and differentiation, and in neurons at advanced stages of differentiation [24]. Both MAGE protein and necdin are devoid of signal sequences and contain a potential transmembrane domain that may function only in association with the transmembrane domain of another protein [25]. It also has been suggested that the expression of the necdin gene is regulated transcriptionally by cell type-specific factors in embryonal carcinoma cells and central neurons during differentiation and development [25].

These observations suggest that MAGE-1 gene expression is limited to a few histologic cell types and that MAGE-1 gene expression is not necessarily linked to neoplastic transformation. Conversely, overexpression of MAGE-1 protein in neoplastic cells may induce such cellular differentiation. The mechanism(s) underlying the regulation of the expression of the MAGE-1 gene in gastric carcinoma cells remains to be determined. Further studies may lead to a better understanding of the physiological and pathological roles of the MAGE family, including MAGE-1, in neoplastic cells.

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